ALK Testing in Non-Small Cell Lung Carcinoma: What Now?

Keith M. Kerr, MD, FRCPath

The rapid evolution in personalized medicine for patients with advanced non—small-cell lung cancer (NSCLC) has brought considerable benefits for those whose tumors are addicted to a particular ongogenic driver, but posed new challenges for pathologists in identifying those cases. Epidermal growth factor receptor (*EGFR*) mutation testing, to underpin the prescription of EGFR tyrosine kinase inhibitors (TKIs), is now a standard of care, where a range of methods may be used to detect and identify the mutation, subject to appropriate validation.[1]

The anaplastic lymphoma kinase (ALK) TKIs are the second class of biomarker-selected "molecularly targeted" treatments to reach maturity in NSCLC therapy, for patients whose tumors harbor an *ALK* gene rearrangement.[2–4] The laboratory identification of *ALK* gene rearrangements has, however, been a rather more contentious issue. These rearrangements are relatively heterogeneous; at least 27 variants have now been described, the vast majority in patients with adenocarcinoma.[5] Most involve an inversion in chromosome 2p, placing a variable length of the echinoderm microtubule-associated protein-like 4 (*EML4*) gene and its promoter upstream of the *ALK* tyrosine kinase domain. Other fusion partners from other chromosomes have the same effect—overexpression of a constitutively activated ALK tyrosine kinase with pro-oncogenic effects. Crizotinib was the "first in class" ALK TKI approved by the U.S. Federal Drug Administration (FDA), in conjunction with a specific companion diagnostic, the Vysis LSI ALK Dual Colour, Break-Apart Rearrangement Probe (Abbott Molecular, Abbott Park, IL). This was the methodology used in the trials that led to the approval of crizotinib; thus, the ALK fluorescence in situ hybridization (FISH) test became the "standard procedure" for patient identification.

FISH testing, and ALK FISH testing in NSCLC in particular, is generally acknowledged as being relatively costly, time-consuming, requires specialized equipment, and requires considerable expertise.[6] Inversions in 2p in particular lead to relatively small physical separation of the two *ALK* fragments, sometimes making the "break apart" difficult to identify. In addition, the requirement for 50 assessable cells to be read, of which at least 15% should show rearrangement for a "positive test," means that as many as 20% of routine diagnostic lung cancer samples requiring testing fall short of this requirement.[6] Although not "FDA approved," alternative methods for detecting *ALK* fusions are attractive.

Reverse-transcriptase polymerase chain reaction (RT-PCR) was the method originally used to identify the *EML4-ALK* gene rearrangement in NSCLC.[7] It is a highly sensitive and specific technique, but it has drawbacks. The ever-increasing number of fusion partners for the *ALK* gene mandates a multiplex RT-PCR approach with multiple specific primers, a relatively "specialist" technique. A greater problem, however, is the availability of adequate quality messenger RNA from tumor samples. Most published studies used fresh-frozen tumor samples. Formalin-fixed, paraffin-embedded samples generally are a poor, unreliable source of messenger RNA. Unfixed cytology type samples have been used in some specialized centers but questions arise over the presence of tumor cells in unexamined test samples, and this approach also poses logistical issues. RT-PCR is not recommended in the College of American Pathologists/International Association for the Study of

Department of Pathology, Aberdeen University School of Medicine, Aberdeen, United Kingdom.
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Address for correspondence: Keith M. Kerr, MD, FRCPath, Department of Pathology, Aberdeen University School of Medicine, Aberdeen Royal Infirmary, Foresterhill, Aberdeen AB25 2ZD, United Kingdom. E-mail: k.kerr@abdn.ac.uk

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Lung Cancer/Association for Molecular Pathology guidelines on ALK testing.[1]

ALK gene rearrangement in NSCLC is associated with a modest elevation in cytoplasmic Alk protein, which may be detected by immunohistochemistry (IHC). Pathologists have long used standard IHC approaches to detect the substantial protein elevations in *ALK*-rearranged anaplastic lymphoma, after which the gene is named. These same techniques were found wanting in NSCLC, through low primary antibody sensitivity but, more importantly, insufficiently sensitive IHC detection systems.[8] The ALK1 antibody clone (Dako, Glostrup, Denmark) fell short in some studies, but many subsequent publications, using either the D5F3 clone (Cell Signalling Technologies, Ventana-Roche, Tucson, AZ) or the 5A4 clone (Leica-Novocastra, Newcastle, UK; Abcam, Cambridge, MA and Cambridge, UK), showed improved results in combination with a range of superior detection systems (reviewed in ref. [6]). IHC has several advantages; it is more widely available, relatively quick and cheap, preserves superior tumor morphology on microscopic examination, and may be assessed on small number of tumor cells. However, approximately 20 published studies describe a range of methodological approaches for antigen unmasking, slide preparation, primary antibody, secondary and amplification stages, not to mention different scoring systems, and definitions of a "positive case."

What does a positive IHC test mean in this debate? Bearing in mind that the current, defining biomarker, at least under FDA rules, is a positive ALK FISH test, the key issue is the ability of a positive IHC test to predict the presence of *ALK* rearrangement as demonstrated by FISH. High-sensitivity detection methods with either 5A4 or D5F3 have demonstrated sensitivity and specificity of ALK IHC, for *ALK* fusion, of 90% to 100%.[6] But still, the heterogeneity of test approaches and a lack of consensus of what constitutes a positive, predictive (for *ALK* fusion) IHC test remain a problem. There are no clinical trials directly validating a positive IHC test as a predictor of response to an ALK TKI, and the FDA has yet to "approve" any particular ALK IHC method for clinical use. The matter was made more complex when the European Medicines Agency approved crizotinib for use, underpinned by "a validated ALK test" without being more specific. Theoretically at least, ALK IHC can be used in Europe to select patients for crizotinib therapy.

In this month's *Journal of Thoracic Oncology*, Wynes et al.[9] present an international, interobserver study on the use of the D5F3 antibody and OptiView detection and amplification system (D5F3-OptiView; Roche-Ventana, Tucson, AZ) in a cohort of 100 adenocarcinomas, 45 of which were positive and 55 negative on ALK FISH testing. There was an excellent interobserver agreement on IHC scoring, and sensitivity and specificity versus ALK FISH positivity were 90% and 95%, respectively. This study maintained excellent performance characteristics, despite samples being sourced from several centers, involving differences in preanalytical variables, and multiple observers. Other reports using the D5F3-OptiView combination, with cases from multiple sources but fewer slide readers,[10,11] report even better figures, effectively 100% each. This staining and scoring methodology can also be consistently performed across multiple centers.[12] The published database,[6] including a multicenter ring study,[13] on the 5A4 antibody also suggests a robust technique, provided the necessary elements are used and rules are followed—this clone is not available as a diagnostic "kit." This takes us some way toward a "real world" testing situation and should help give oncologists confidence in IHC as a reliable ALK biomarker diagnostic. The author is unaware of any similar published studies of ALK FISH testing.

There are, of course, some issues with ALK IHC. Testing outcomes may be compromised by vagaries of preanalytical handling, but so too for FISH testing. Some reports, many anecdotal, of discrepant ALK IHC or FISH tests (IHC+/FISH− or IHC−/FISH+) probably reflect technical failures in one of the tests in many instances[14] because of pre-cut section deterioration, fixation/processing/handling issues, lack of tumor in test sections, slide misinterpretation, clerical error, and so on. Nonetheless, true biological discrepancies may occur: A rearrangement may be present but may not be transcribed or translated; such cases may be associated with other driver mutations.[15] Alk protein may be elevated in cases which lack gene rearrangement. This may be associated with *ALK* gene amplification.[16]

Interpretation of IHC is an important factor. The detection systems often used with the 5A4 clone deliver variable staining intensity, allowing for the traditional four-tier (0, 1+, 2+, and 3+) scoring system. There is variability in the reported significance of a 1+ and even a 2+ stain, but 0 and 3+ scores have a very high negative and positive predictive value, respectively, for FISH positivity. Thus, the definition of a positive predictive 5A4-based IHC test has been elusive and accounts for some of the "poor performance" reported for some test approaches. The detection and amplification system used by Wynes et al.[9] and others[10–12] uses tyramide-based chemistry that generates a relatively large amount of chromogen (color stain on the tissue section) per molecule of bound D5F3 antibody.[17] This shifts the dynamic range of the staining result, away from a graduated range of staining intensity to a more binary, on/off situation. This principle has been well discussed elsewhere.[18] Wynes et al.,[9] in line with others' experience,[6] however, indicate that the OptiView methodology does not give a completely binary "present or absent" signal, but it is close to being so. Some cases undoubtedly show variable staining intensity, but H-scores do tend toward the extremes of the 0 to 300 range.[17] Some cases show background staining that could lead to a false-positive call. In the author's experience, such "borderline" cases are more likely to be either cytology cell block cases or cases from outside centers but have never been ALK FISH positive. This test may show staining in alveolar macrophages, bronchial gland cells, neural tissue, and occasional lymphoid cells. These are of crucial importance when interpreting difficult samples with few tumor cells admixed with non-neoplastic pulmonary tissue. The IHC appearances of the D5F3-OptiView technique are different from other detection systems, and training on scoring helps interobserver agreement.[12] This technique, with careful observation of what constitutes a "positive," supported by these latest data presented here, seems to offer a reliable, standardized ALK IHC test.

Given that IHC can be a reliable ALK testing strategy, how should it be used? Although there are case reports of ALK IHC-positive, FISH-negative patients responding to crizotinib,[19,20] there are no phase 3 clinical trials supporting ALK IHC as a primary, therapy-selecting biomarker. However, given that the overall response rate of cohorts selected by FISH is 53% to 65%, and that 17.5% of FISH-positive patients in one trial showed no disease control at all,[3] it would be very surprising, given the close correlation between ALK IHC and FISH test outcomes, if ALK IHC positivity were not associated with similar clinical responses. The strong correlation between IHC and FISH negativity, and the pros and cons of IHC and FISH already mentioned above have underpinned the use of ALK IHC as a screening tool to eliminate many candidate cases from FISH testing where ALK IHC is negative. This approach is supported by the published data and the College of American Pathologists/International Association for the Study of Lung Cancer/Association for Molecular Pathology guidelines. Although there may be debate as to whether 1+ IHC-positive cases from sensitive 5A4-based studies should be FISH tested or not, IHC screening is still beneficial. With the D5F3-OptiView technique, the borderline cases would seem to be fewer, and anecdotal evidence suggests that they will not be ALK rearranged, but more data will be welcome.

Assuming analytical problems are solved, the technology becomes widely available, and requirements on quality and quantity of extracted DNA can be met[21,22]; will next-generation sequencing technology make the ALK IHC/FISH debate irrelevant? A reliable method to detect *ALK* gene rearrangements in DNA as part of a multiplex genomic screen would be very welcome. These findings would, however, still require validation in trials, as a method to detect patients likely to respond to ALK TKIs. As already mentioned, it is the abnormally expressed protein, not the abnormal DNA sequence, which exerts oncogenic effects, and the protein is the target of the drug. There are suggestions and anecdotal reports that patients with *ALK* rearrangement (FISH or RT-PCR positive) but no protein expression (IHC negative) do not respond so well to ALK TKIs (personal communication, Yasushi Yatabe, MD, Aichi Cancer Centre, Nagoya, Japan).[23] This may be part of the reason why, in the published trials, the response rates for ALK FISH-positive cohorts are "only" 53% to 65%. We face the intriguing possibility of the IHC test becoming the primary biomarker for ALK TKIs, perhaps with next-generation sequencing being used as the screening step.

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